Loss of sodium chloride co-transporter impairs the outgrowth of the renal distal convoluted tubule during renal development

Schnoz, Christina ; Carrel, Monique ; Loffing, Johannes

Abstract: BACKGROUND: oss-of-function mutations in the sodium chloride (NaCl) co-transporter (NCC) of the renal distal convoluted tubule (DCT) cause Gitelman syndrome with hypokalemic alkalosis, hypomagnesemia and hypocalciuria. Since Gitelman patients are usually diagnosed around adolescence, we tested the idea that a progressive regression of the DCT explains the late clinical onset of the syndrome. METHODS: CC wild-type and knockout (ko) mice were studied at Days 1, 4 and 10 and 6 weeks after birth using blood plasma analysis and morphological and biochemical methods. RESULTS: lasma aldosterone levels and renal renin messenger RNA expression were elevated in NCC ko mice during the first days of life. In contrast, plasma ion levels did not differ between genotypes at age 10 days, but a significant hypomagnesemia was observed in NCC ko mice at 6 weeks. Immunofluorescent detection of parvalbumin (an early DCT marker) revealed that the fractional cortical volume of the early DCT is similar for mice of both genotypes at Day 4, but is significantly lower at Day 10 and is almost zero at 6 weeks in NCC ko mice. The DCT atrophy correlates with a marked reduction in the abundance of the DCT-specific Mg2+ channel TRPM6 (transient receptor potential cation channel subfamily M member 6) and an increased proteolytic activation of the epithelial Na+ channel (ENaC). CONCLUSION: fter an initial outgrowth, DCT development lags behind in NCC ko mice. The impaired DCT development associates at Day 1 and Day 10 with elevated renal renin and plasma aldosterone levels and activation of ENaC, respectively, suggesting that Gitelman syndrome might be present much earlier in life than is usually expected. Despite an early downregulation of TRPM6, hypomagnesemia is a rather late symptom.

DOI: https://doi.org/10.1093/ndt/gfz172

Loss of NCC impairs the outgrowth of the renal distal convoluted tubule (DCT) during renal development

Christina Schnoz[1] · Monique Carrel[1] · Johannes Loffing[1,2]

[1]Institute of Anatomy, University of Zurich, 8057 Zurich, [2]National Centre of Competence in Research "Kidney.CH", University of Zurich, 8057 Zurich

Corresponding author

Johannes Loffing

University of Zurich, Institute of Anatomy

Winterthurerstrasse 190

CH-8057 Zurich

Switzerland

Phone: +41 (0) 44 635 53 20

Fax: +41 (0) 44 635 57 02

Email: johannes.loffing@anatomy.uzh.ch

Abstract

Loss-of-function mutations in the NaCl co-transporter (NCC) of the renal distal convoluted tubule (DCT) cause Gitelman syndrome with hypokalemic alkalosis, hypomagnesemia and hypocalciuria. Since Gitelman patients are usually diagnosed around adolescence, we tested the idea that a progressive regression of the DCT explains the late clinical onset of the syndrome. NCC wt and ko mice were studied at day 1, 4, 10 and 6 weeks after birth. Plasma aldosterone levels and renal renin mRNA expression were elevated in NCC ko mice already during the first days of life. In contrast, plasma ion levels did not differ between genotypes at age 10 days, but a significant hypomagnesemia was observed in NCC ko mice at 6 weeks. Immunofluorescent detection of parvalbumin (an early DCT marker) revealed that the fractional cortical volume of the early DCT is almost similar for mice of both genotypes at day 4, but gets significantly lower at day 10 and is almost zero at 6 weeks in NCC ko mice. The DCT atrophy correlates with a marked reduction in the abundance of the DCT-specific Mg^{2+} channel TRPM6 and an increased proteolytic activation of the epithelial Na^{+} channel (ENaC). Thus, after an initial outgrowth, DCT development lags behind in NCC deficient mice. The impaired DCT development associates already at day 10 with elevated renal renin and plasma aldosterone levels and an activation of ENaC, suggesting that Gitelman syndrome might be present much earlier during life than usually expected. Despite an early downregulation of TRPM6, hypomagnesemia is a rather late symptom.

Keywords

Gitelman syndrome, renal development, DCT development, NaCl cotransporter (NCC), transient receptor potential melastatin type 6 (TRPM6)

Running title

Early impact of NCC deficiency

Introduction

The renal distal convoluted tubule (DCT) plays a crucial role in the maintenance of extracellular fluid volume, regulation of arterial blood pressure and electrolyte homeostasis. Sodium reabsorption in the DCT constitutes 5-10% of the entire sodium absorption along the nephron and is primarily mediated by the thiazide-sensitive sodium chloride cotransporter (NCC) [1], which is uniquely expressed in the DCT. NCC is the target of thiazide and thiazide-like diuretic drugs that are commonly used for long-term treatment of arterial hypertension [2]. The importance of the DCT and NCC to human physiology is also confirmed by Gitelman syndrome, a genetic disease caused by loss-of-function mutations in NCC leading to an autosomal recessive renal tubulopathy characterized by hypokalemic alkalosis, hypomagnesemia, hypocalciuria, mild renal salt wasting and normal to low arterial blood pressure [3,4].

Patients suffering from Gitelman syndrome are considered to be rarely symptomatic (muscle weakness and spasms, fatigue due to potassium and magnesium depletion) during childhood and the disease is generally diagnosed during adolescence or early adulthood [5]. The late clinical onset of Gitelman syndrome is opposed to Bartter syndrome, which represents a group of inherited renal tubulopathies related to mutations in various genes (i.e. the Na-K-2Cl cotransporter NKCC2, the K^+ channel ROMK, the Cl^- channel ClC-Kb or its subunit Barttin BSND, and the Calcium-sensing receptor CSR), which finally lead to an impaired NaCl reabsorption in the thick ascending limb of Henle's loop [6]. Patients with Bartter syndrome suffer from severe electrolyte disturbances with tetany, polyuria and volume depletion that get usually symptomatic already in the prenatal period [7]. The reasons for the rather late onset of the clinical manifestation in most Gitelman patients remains elusive.

Schultheis et al [8] had generated an NCC null mutant mouse model. These mice show a renal phenotype which recapitulates many of the findings in human Gitelman patients including hypomagnesiemia, alkalosis and hypocalciuria [8,9]. Moreover, distinctive alterations in the epithelial structure of the DCT, showing a marked atrophy of the DCT due to an almost complete absence of the early DCT portion (DCT1) was demonstrated in NCC ko mice [8]. The loss of a significant portion of the DCT epithelium was accompanied by a marked reduction of the abundance of the DCT-localized apical Mg^{2+} channel TRPM6, which was suggested to explain the renal Mg^{2+} wasting seen in the NCC-deficient mice and patients with

Gitelman-syndrome [10]. Moreover, adult NCC-deficient mice show a marked structural hypertrophy of the connecting tubule (CNT), which goes along with an up-regulation of the epithelial Na^+ channel ENaC in the kidney [9,11], which may not only compensate for the loss of NCC activity in the DCT, but may also contribute to the susceptibility of the NCC-deficient mice [12] and Gitelman patients for hypokalemia [3,4].

The marked epithelial remodeling of the distal tubule described above may constitute a morphological basis for the pathophysiology of Gitelman-syndrome. Although the underlying mechanism for the marked structural atrophy of the DCT is unclear, the loss of the DCT epithelium likely reflects the altered ion transport activity due to the loss of NCC. Consistently, also the inhibition of NCC with thiazide-diuretics causes DCT cell apoptosis [13]. Interestingly, a loss of carbonic anhydrase II (CADII) in CADII-ko mice, is also accompanied by a depletion of the cells usually expressing this particular gene (i.e. the intercalated cells in the renal collecting system) [14]. The loss of intercalated cells in CADII deficient mice starts at about day 11 after birth with a bisection of intercalated cells by 6 weeks of age and an almost complete loss of intercalated cells by age of 3-5 months [15]. Thus, in CADII ko mice, the depletion of intercalated cells occurs quite late. The time course of the occurrence of the DCT atrophy in NCC deficient mice is not defined. This study addresses the question whether a rather late onset of the DCT atrophy may explain the late clinical presentation of Gitelman syndrome. To reach this end, we evaluated DCT development and the expression of key distal tubule ion transporters and channels in NCC wt and ko mice at days 1, 4, and 10 and week 6 after birth.

Material and Methods

Animals

Generation of NCC deficient mice has been described previously [8]. All mice used in this study had a C57/Bl6 background. Animals were bred in a standard, non-specific pathogen free animal facility. All experiments involving living animals were conducted in accordance with Swiss laws and approved by the veterinary administration of the Canton of Zurich, Switzerland.

Tissue processing

Mice aged 1, 4 or 10 days were anesthetized with isoflurane and killed by decapitation. Kidneys were then immediately removed for subsequent analysis. For immunohistochemistry, kidneys were immersion fixed with paraformaldehyde (PFA) 3% for 12-16 hours, then incubated in 0.1 M phosphate buffer (pH 7.4, 300 mOsm) for 4 hours and finally embedded in paraffin. For RNA extraction and western blot analysis, kidneys were frozen in liquid nitrogen and stored at -80°C until future use. *Mice aged 6 weeks* were anesthetized with isoflurane and the kidneys were fixed by retrograde abdominal aortic perfusion of 3% PFA followed by rinsing with 0.1 M phosphate buffer (pH 7.4, 300 mOsm) and paraffin embedding. For RNA extraction and western blot analysis, perfusion was performed with PBS via the left ventricle of the heart, kidneys were removed, frozen in liquid nitrogen and then stored at -80°C.

Immunohistochemistry

Paraffin tissue sections of 4 µm were deparaffinized using Histo-clear and rehydrated through serial ethanol washes (Pathisto AS-2 automatic slide stainer). For epitope retrieval, slides were heated in 10 mM citrate buffer (pH 6.0) in a microwave (98°C, 10 min). After blocking for 10 min with 10% normal goat serum in in PBS-1% BSA, the sections were incubated overnight in a humidified chamber at 4°C with the primary antibodies, diluted in PBS-1% BSA. The primary antibodies and dilutions used for immunohistochemistry are shown in supplementary table 1. Secondary antibodies (Cy3-conjugated goat-anti-rabbit IgG, catalogue code 111-165-144, dilution 1/1000 and FITC-conjugated goat-anti-mouse IgG, catalogue code 115-095-068, dilution 1/100, both from Jackson Immuno Research Laboratories, West Grove, PA) were applied to the slides for 2 hours at room temperature, diluted in PBS-1% BSA. After final washing with PBS, sections were mounted using DABKO-glycergel and coverslips. Images were

acquired at a Leica DM6000 B fluorescence microscope on Leica DFC350 FX fluorescence monochrome digital camera (Leica Microsystems, 35578 Wetzlar, Germany).

PCNA Labeling Index

PCNA-positive cell nuclei were detected in paraffin sections using immunohistochemistry as described above. At the microscope, DCT1 segments were identified by parvalbumin-positive immunostaining. The number of all PCNA-positive DCT1 cell nuclei within a kidney cross section was counted and expressed as percentage of all DAPI-stained cell nuclei in the DCT1 segments.

TUNEL Assay

Apoptotic cell nuclei were detected in paraffin sections using In Situ Cell Death Detection Kit, Fluorescein (Roche), according to the manufacturer's instructions.

Morphometric measurements

The fractional tubular volume of DCT segments was assessed using a planimetric point-counting method according to a previously described protocol [16]. In brief, PFA-fixed kidneys were cut into halves perpendicular to their longitudinal axis. After embedding in paraffin, the kidneys were cut into 4 µm thick sections and stained with polyclonal antibodies against NCC (NCC wt samples) and parvalbumin (NCC wt and NCC ko samples) as described above. Overviews were taken from each section at the microscope using the 10x objective. DCTs were identified according to their specific antibody-staining pattern. Morphometric analysis was then performed with a computerized image analysis system (Stereo Investigator, MBF Bioscience, Williston USA). A transparent grid with a distance of 50 µm between lines was electronically superimposed on the micrographs. Cortical areas extending between the renal capsule and the outer medullary boundary, identified by arcuate arteries, were evaluated. The intersections of grid lines falling on the total cortex area, and those intersections falling on DCT segments were counted. The proportion of DCTs in the cortex was calculated as the percentage of DCT counts versus the total number of cortex counts and expressed as the fractional cortical volume of the DCT.

RNA isolation and quantitative PCR

Kidneys were harvested as described above. Total RNA from kidney tissue was isolated using Promega kits (ReliaPrep™ RNA Tissue Miniprep System for the kidneys of 1, 4, and 10 day old mice; SV Total RNA Isolation System for the kidneys of 6-week-old mice), according to the manufacturer's protocol. 500 ng of total RNA was used for the generation of cDNA using Promega kit (GoScript™ Reverse Transcription System) as per the manufacturer protocol. This cDNA was further diluted to 1:5 and expression levels of parvalbumin, TRPM6 and renin were quantified by real-time quantitative PCR using Light Cycler II 480 (Roche). Relative gene expression was determined after normalization to ribosomal RNA gene expression. The primers used for RT-qPCR are listed in supplementary table 2.

Plasma ion measurement

Blood collection – In 6-week-old mice, blood was sampled by puncture of the inferior vena cava of isoflurane anesthetized animals. In 10-day-old pups, trunk blood was collected after anesthesia with isoflurane and decapitation. Whole blood was centrifuged for 10 min at 6000 rpm, plasma was removed and frozen at -80°C until further use. *Plasma Na^+, K^+ and Ca^{2+} measurement* – In 6-week-old mice, blood ions (Na^+, K^+, Ca^{2+}) were measured with the ABL80Flex Blood Gas Analyzer (Radiometer, Copenhagen, Denmark). In 10-day-old pups, plasma electrolytes (Na^+, K^+, Ca^{2+}) were measured by flame photometry (EFOX 5053, Eppendorf). *Plasma Mg^{2+} measurement* – Plasma Mg^{2+} measurements were performed by the Zurich Integrative Rodent Physiology (ZIRP) using photometric methods (UniCel® DxC 800).

Aldosterone measurement

Blood of 10-day-old pups was collected as described above. Plasma aldosterone was measured by an ELISA assay (Aldosterone ELISA Kit, Cayman chemical, Item No. 501090) according to the manufacturer's instructions.

Western blot Analysis

Sample preparation - Kidneys were homogenized in ice-cold lysis buffer (Mannitol 200 mM, HEPES 80 mM, KOH 41mM) containing protease inhibitor (Complete Ultra, Roche) and phosphatase inhibitor (PhosSTOP, Roche). Tissue homogenization was performed using MagNA Lyser Green Beads (Roche) and Precellys 24 tissue homogenizer (Bertin Instruments), 2 x 20 sec (5000 rpm). The homogenized samples were centrifuged for 10 min (4600 rpm) and

the protein-containing supernatant was removed and stored at -80°C. *SDS-Page* – Equal amounts of protein (25-50 µg) from kidneys of wt and ko mice were denatured in Laemmli Buffer (SDS, β-mercaptoethanol, bromophenol blue, glycerol and 0.5M Tris-HCl buffer, pH 6.8) and loaded on 8-12% polyacrylamide gels. *Transfer and antibody incubation* – After electrophoretic separation, proteins were transferred to a nitrocellulose membrane. Equal protein loading and electrophoretic transfer were confirmed by using REVERT Total Protein Stain (LI-COR Biosciences) and visualization of protein bands with an Odyssey IR imaging system (LI-COR Biosciences) prior to antibody incubation. The membrane was blocked for 10 min with Odyssey Blocking solution (LI-COR Biosciences) and then incubated at 4°C for 16 hours with the primary antibodies (table 1 of supplementary data) diluted in Odyssey Blocking buffer : PBS (1:5). After repeated washes with PBS, blots were incubated at room temperature for 2 hours with secondary antibodies (goat-anti-rabbit IRDye 800, product number 926-32211 and goat-anti-mouse IRDye 680, product number 926-32220, 1:20'000, both from LI-COR Biosciences), in Casein Blocking solution : H2O (1:10). Quantification of immunoreactive bands was carried out with the Odyssey IR imaging system and software (LI-COR Biosciences) and normalized for the total protein signal obtained for each sample after the electrophoretic transfer (see above).

Generation of affinity purified rabbit-anti-mouse TRPM6 antibody

The new antibody against TRPM6 was obtained by immunizing rabbits (Pineda Antikörper-Service, Berlin, Germany) with keyhole limpet-hemocyanin-coupled synthetic peptides corresponding to the amino acid sequence within mouse TRPM6 (NH2-CERDKNRSSLEDHTRL-COOH). After affinity-purification against the immunizing peptide (Pineda Antikörper Service), the antibody was characterized by using standard Western blotting with wildtype mouse kidney samples (as described above). Pre-incubation of the antiserum (concentration 1:2000 in PBS) with the peptide used for immunization (concentration 20 µg/ml in PBS) for 2 hours at room temperature with subsequent centrifugation (13'000 rpm, 10 min) completely inhibited the specific binding of the antibody to the nitrocellulose Western blot membrane (supplementary figure 1).

Statistical Analysis

Unpaired two-tailed t-test, one-way ANOVA and two-way ANOVA were used to compare the groups (GraphPad Prism, Version 8.0.2). Data are given as means +/- SD (in tables) and means +/- SEM (in figures). Values were considered as significantly different when $p < 0.05$.

Results

Overt hypomagnesemia in NCC ko mice at age 6 weeks

At the age of 10 days, the plasma concentrations of Na^+, K^+, Ca^{2+} and Mg^{2+} were similar for NCC wt and NCC ko mice (table 1). For both groups of mice, the measured plasma K^+ levels were rather high, likely reflecting some artificial hemolysis due to the difficult blood sampling in these young animals. At the age of 6 weeks, the plasma concentrations of Na^+, K^+ and Ca^{2+} were all in the normal range and similar between NCC wt and NCC ko mice. However, plasma Mg^{2+} levels were significantly lower in NCC ko than in NCC wt mice. The different plasma Ca^{2+} concentrations of 10-days and 6-weeks-old mice are likely related to technical reasons (i.e. different methods used for blood collection and plasma Ca^{2+} measurements in 10-days vs. 6-weeks old mice).

Increased renal renin expression and hyperaldosteronism in NCC ko mice at age 10 days

Already at the age of 1 day, the mRNA expression levels for renin were significantly higher in kidneys of NCC ko mice than in kidneys of NCC wt mice (figure 1A). Renin mRNA expression tended to be higher in NCC KO versus NCC wt mice also at the age of 4 days, 10 days and 6 weeks. However, the differences did not reach statistical significance anymore (figure 1A). Nevertheless, consistent with a continuous activation of the RAAS, plasma aldosterone levels were found to be increased in NCC ko animals at the age of 10 days when compared to the corresponding levels in NCC wt mice (figure 1B). Due to the limited amounts of blood that could be collected from very young pups, plasma aldosterone levels could not be measured at the age of 1 and 4 days.

DCT outgrowth in NCC wt mice occurs mainly during the first 10 postnatal days

Immunofluorescent staining for the DCT-specific NaCl cotransporter (NCC) showed an increase of NCC expression in the renal cortex of NCC wt mice during the first 10 days of life (figure 2A). Consistently, morphometric analysis revealed that the fractional cortical tubular volume of the DCTs gets progressively higher until day 10 after birth (figure 2B). At the age of 10 days, the fractional cortical tubular volume for the DCT reaches ~7%, which is similar to the value seen in adult NCC wt mice. In parallel to the outgrowth of the DCT, the NCC mRNA expression (figure 2C) and the NCC protein expression (figure 2D) increased up to day 10 and remained then at the same level. Although not statistically significant, the abundance of NCC

protein tended to increases further after day 10, suggesting a posttranscriptional mechanism for the additional increase in NCC abundance from day 10 to week 6.

Impaired postnatal development of the DCT1 in NCC ko mice

For obvious reason, we could not use the detection of NCC to identify DCTs in NCC ko mice. Therefore, we relied, as in previous studies, on the detection of the calcium- and magnesium binding protein parvalbumin (PV), which is a marker for the early DCT (DCT1) [9]. Immunostaining of kidneys from 10 day old wildtype mice confirmed the reliability of this approach for the developing mouse kidney. As in adult kidneys, PV expression in kidneys of 10 days old pups started precisely at the transition from the NKCC2-positive TAL to the NKCC2-negative early DCT (DCT1) (supplementary figure 2A), which was also characterized by a very low abundance of calbindin (CB). Parvalbumin abundances ceased then abruptly at the transition from the weak CB-positive early DCT (DCT1) [17] to the strong CB-positive late DCT (DCT2). DCT1 and DCT2 are both NCC-positive (supplementary figure 2B). Interestingly, the morphometric quantification of the fractional cortical volume of PV-positive tubules (i.e. DCT1) versus the one of NCC-positive tubules (i.e. DCT1 and DCT2) revealed that the ratio of DCT1 to DCT2 various with age. As observed previously in the rat kidney [18], the developing DCT (day 1 and 4 after birth) is mainly composed by DCT2 cells, while at later stages of development (day 10 and week 6 after birth), the DCT1 cells appear to dominate (figure 3). The reason for this developmental switch from a DCT2 predominance to a DCT1 predominance is unclear.

When we then compared kidneys from NCC wt and NCC ko mice, we found that the abundance of PV-positive tubules (DCT1s) in the renal cortex increases progressively with age in NCC wt mice. In contrast, PV-positive tubules are barely visible in kidneys of NCC ko mice at all analyzed time points (figure 4A, 4B). Morphometric analysis confirmed this observation. In NCC wt mice, the fractional cortical volume of PV-positive tubules increases with age suggesting a constant outgrowth of this segment. In contrast, after an initial minor outgrowth (until day 4), the development of the DCT1 appears to cease in kidneys of NCC ko mice (Figure 4C). Consistent with an impaired outgrowth of the DCT1, PV gene expression tended to be lower in the kidneys of NCC ko mice than in kidneys of NCC wt animals (Figure 4D), though differences reached statistical significance only at 6 weeks of age.

Reduced DCT cell proliferation in NCC ko mice at age 4 days and 10 days

To test whether a reduced cell proliferation may explain the impaired outgrowth of the DCT1 in NCC ko mice, we used immunodetection of the proliferating cell nuclear antigen (PCNA), which is a reliable cell proliferation marker. As shown in figure 5, the number of PCNA-positive cell nuclei is drastically lower in PV-positive DCT1 cells of NCC ko mice than in PV-positive DCT1 cells of NCC wt animals for both day 4 and day 10 of age.

Sporadic DCT cell apoptosis in NCC ko mice at age 4 days and 10 days

As the morphometric analysis suggested that the fractional volume of the DCT1 does even decrease from day 4 to day 10 in NCC ko mice, we analyzed whether the frequency of apoptotic cell death in the DCTs of NCC ko mice might be increased at this age. For the detection of apoptosis, we used the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay and combined it with immunostaining for PV to identify early DCT (DCT1). Apoptotic cell nuclei were detected very rarely in developing renal tubules and the interstitium of the nephrogenic zone in both groups of mice at the age of 4 and 10 days (supplementary figure 4). Only one apoptotic DCT cell in total was detected in the analyzed NCC wt kidney samples (one paraffin section each from 3 wt mice at the age of 4 days and 3 wt mice at the age of 10 days) and five apoptotic DCT cells were found in NCC ko kidney samples (one paraffin section each from 3 ko mice at the age of 4 days and 3 ko mice at the age of 10 days).

Reduced protein expression of the DCT-specific Mg^{2+} channel TRPM6 in NCC ko mice

Western blot analysis revealed a significant reduction in the protein expression of the DCT-specific Mg^{2+}-channel TRPM6 in NCC ko versus NCC wt at age of 1 day, 4 days, 10 days and 6 weeks (figure 6A+B, supplementary figures 4+5). In contrast, the abundance of the Ca^{2+} reabsorbing transient receptor potential vanilloid 5 (TRPV5) Ca^{2+} channel, which is expressed in DCT2 and CNT, was similar in NCC wt versus ko mice (figure 6A+B).

Compensatory upregulation of the epithelial Na^{+} channel (ENaC) in response to loss of NCC

Protein expression analysis for α-, β- and ɣ-ENaC revealed a significant upregulation of the cleaved forms of α- and/or ɣ-ENaC at the age of 4 days, 10 days and 6 weeks, while β-ENaC protein abundance was downregulated at day 4 and day 10 (figure 6A+B, supplementary figures 4+5).

Discussion

The renal distal convoluted tubule (DCT) has a remarkable structural plasticity. The DCT responds to an increased tubular workload with a pronounced epithelial hypertrophy, while a reduced transport activity in the DCT is associated with an epithelial hypotrophy [19]. Previous studies on adult mice had shown that the genetic deletion of the thiazide-sensitive NaCl cotransporter NCC is associated with marked epithelial atrophy of the early DCT (DCT1). In the present study, we addressed the question whether NCC deficiency impairs the outgrowth of the distal convoluted tubule already during renal development or whether the DCT atrophy occurs at later stages of life due to a regression of the already developed DCT.

In general, no grave phenotypic features such as growth retardation, severe electrolyte disturbances or metabolic abnormalities were observed in NCC deficient mice, neither during the developmental period nor at adulthood. However, our study shows that NCC ko mice lack a significant outgrowth of the DCT during early perinatal development. The disturbed DCT growth during renal development in NCC ko mice results in a marked DCT atrophy at adulthood. We showed previously that the DCT atrophy concerns mainly the early DCT (DCT1) rather than the late DCT (DCT2) [9]. We did not address differential effects on DCT1 and DCT2 morphology in the present study. However, our observation that the DCT1-specific marker protein parvalbumin is much more vanished from kidneys of NCC ko mice than the DCT1 and DCT2-marker protein TRPM6 indicates that the DCT1 is preferentially affected already during renal development. The structural atrophy of the DCT is presumably accompanied by changes in DCT tubular function and may explain some of the clinical features of Gitelman syndrome. Signs of volume depletion with increased renal renin expression and hyperaldosteronism, common symptoms in human Gitelman patients and previously also described in adult NCC ko mice [8,12], were already present in mice at the age of 1 day and 10 days, respectively. The very high up-regulation of renal renin expression at day 1 is surprising, but might reflect the more difficult adaptation of newborn NCC ko mice to postnatal life. Perhaps, direct after birth, the NCC ko mice lose a lot of sodium until the strong activation of the RAAS system leads to a compensatory up-regulation of ENaC and possibly other Na+ retaining mechanism. Consistent with this idea, our immunoblot data did not show clear evidence for a significant up-regulation of ENaC in NCC ko mice at day 1 after birth. However, at day 4, day 10 and week 6, proteolytic cleavage of ENaC subunits was observed, which is indicative for an ENaC activation [20].

Overall, our data strongly suggests that the renal tubular dysfunction leading to renal sodium wasting and volume contraction manifests in NCC ko mice already at very early age.

Hypomagnesemia is another key characteristic of Gitelman syndrome [3,4]. Interestingly, low plasma magnesium levels were only found in adult NCC ko mice, but not in NCC ko 10-days-old pups, even though the expression of the DCT1-specific Mg^{2+} channel TRPM6 was significantly decreased in NCC ko mice at both the age of 10 days and 6 weeks. Most probably, NCC ko pups are born with a normal body magnesium content. We hypothesize that magnesium wasting in NCC ko only starts postnatally and a prolonged time period is necessary to establish a relevant magnesium depletion. Additionally, the transition from breast feeding nourishment to lab chow may also contribute to the development of hypomagnesemia. It would be interesting to know the situation in newborn human Gitelman patients. Given that most human patients do not become symptomatic during early childhood [5], one can speculate that plasma magnesium is also not, or at least not drastically, reduced at neonate age, as demonstrated in this NCC ko mouse model.

Hypokalemia is another cardinal feature in human Gitelman patients [3,5]. Interestingly and consistent with previous studies on adult mice [8], the NCC ko mice did not show any hypokalemia at any of the analyzed ages. This difference between human Gitelman patients and NCC ko mice may reflect real species differences but could be also related to the exceedingly higher K^+ intake of mice compared with humans. On the standard mouse chow with 0.8% K^+, adult mice approximately ingest 30-40 mg potassium [21]. The average daily K^+ intake of adult humans is in the range of 2,9 g for men and 2,3 g for women [22]. For 25 g mouse and a 70 kg human this equals to a daily K^+ intake of 1.2-1.6 g potassium / kg BW in the mouse and 0.04-0.03 g/kg BW for a human. Thus, normalized for body weight, mice have an about 40 times higher daily K^+ intake than humans. The idea that the high K^+ intake on standard diet may protect NCC ko mice from hypokalemia is supported by the observation that the mice become rapidly hypokalemic when K^+ intake is restricted [12].

Gitelman patients and NCC ko mice are hypocalciuric [3,4,8]. Likewise, thiazide diuretics reduce urinary Ca^{2+} excretion [23]. Although NCC inhibition with thiazides increases calcium uptake by DCT cells [24,25], micropuncture and biochemical studies on NCC ko mice and thiazide-treated mice indicated that the reduced renal calcium excretion in these mice is

due to an enhanced passive paracellular calcium reabsorption in the proximal tubule rather than due to an increased transcellular calcium transport in the distal tubule [9,23].

The loss of NCC leads to major structural remodeling of the DCT, which includes a reduction of the individual cell height and of the tubular length of the DCT [19]. Our present study suggests that the drastic shortening of the DCT in adult NCC deficient mice is most probably the consequence of an incomplete outgrowth of the DCT1 during early perinatal kidney development, indicated by the significantly lower rate of PCNA-detected DCT1 cell proliferation in NCC ko mice compared to NCC wt mice for both age day 4 and day 10 after birth. Apparently, a few parvalbumin-positive tubuli are formed in both NCC wt and NCC ko mice at the age of 4 and 10 days. However, this minor outgrowth of the DCT discontinues in the kidneys of NCC ko mice. Even though apoptosis was more frequently observed in NCC ko mice, apoptotic events occurred only rarely in both genotypes and its significance remains unclear. Finally, the early DCT is almost completely absent from the kidneys of 6 weeks old NCC ko mice. This marked structural atrophy diminishes the available epithelial cell surface area along the DCT and may hence explain the reduced TRPM6 abundance and renal Mg^{2+} wasting in NCC ko mice reported in previous [8] and in this study. Likewise, pharmacological inhibition of NCC by prolonged treatment with thiazides (e.g. hydrochlorothiazide) or thiazide-like diuretics (e.g. metolazone) often causes hypomagnesemia in human [26,27] and mouse [19,23], which is also thought to be related to a downregulation of TRPM6 at the mRNA and protein level. We do not know about the effect of the genetic loss or pharmacological inhibition of NCC on the structural integrity of the DCT in humans. However, the observation of an almost complete loss of the early DCT in NCC-deficient mice [9] and of massive apoptotic death in the early DCT of thiazide-treated rats [13] suggests that the loss of epithelial surface area in the DCT may explain the hypomagnesemia observed in Gitelman patients and humans treated with thiazide-diuretics.

In summary, the genetic loss of NCC leads to remarkable structural and functional changes in mouse kidneys. Our data indicate that NCC deficiency mainly impairs the outgrowth of the developing DCT rather than causes a regression of already formed DCTs. Consistently an activation of the renin-angiotensin-aldosterone system with a compensatory up-regulation of ENaC and a downregulation of TRPM6 is detectable already quite early during life-time. Together with the observation in humans that the clinical manifestation and onset

of Gitelman syndrome varies considerably [28,29,30], one can speculate that signs of Gitelman-syndrome are already present at very young age but are not diagnosed, as laboratory testing for plasma magnesium and potassium as well as urinary calcium and magnesium excretion is not routinely performed in children.

Acknowledgments

The authors thank Dominique Loffing-Cueni, Dario Mattle, and Eszter Banki for the initial characterization of the TRPM6 antibody. The TRPV5 antibody was a kind gift of Olivier Bonny (Lausanne). The technical assistance of Michèle Heidemeyer is kindly acknowledged.

Grants

Christina Schnoz is member of the UZH MD/PhD program. Johannes Loffing is supported by research funds from the Swiss National Centre for Competence in Research “Kidney.CH” and by a project grant from the Swiss National Science Foundation (310030_143929/1).

Disclosures

Authors have nothing to disclose.

References

[1] McCormick JA, Ellison DH. Distal Convoluted Tubule. *Compr Physiol* 2015; 5:45-98

[2] Ellison DH, Loffing J. Thiazide effects and side effects: insights from molecular genetics. *Hypertension* 2009; 54(2):196-202

[3] Gitelman HJ, Graham JB, Welt LG. A new familial disorder characterized by hypokalemia and hypomagnesemia. *Trans Assoc Am Physicians* 1966; 79:221-235

[4] Simon DB, Nelson-Williams C, Johnson Bia M, et al. Gitelman's variant of Bartter's syndrome, inherited hypokalaemic alkalosis, is caused by mutation sin the thiazide-sensitive NaCl cotransporter. *Nat Genet* 1996; 12:24-30

[5] Knoers N, Levtchenko E. Gitelman syndrome. *Orphanet J Rare Dis* 2008; 3:22

[6] Da Silva Cunha T, Pfeferman Heilberg I. Bartter syndrome: causes, diagnosis, and treatment. Int J Nephrol Renovasc Dis 2018; 11:291-301

[7] Al Shibli A, Narchi H. Bartter and Gitelman syndromes: Spectrum of clinical manifestations caused by different mutations. *World J Methodol* 2015; 5(2): 55-61

[8] Schultheis PJ, Lorenz JN, Meneton P, et al. Phenotype resembling Gitelman's syndrome in mice lacking the apical Na+-Cl- cotransporter of the distal convoluted tubule. *J Biol Chem* 1998; 273:1-6

[9] Loffing J, Vallon V, Loffing-Cueni D, et al. Altered renal distal tubule structure and renal Na+ and Ca2+ handling in a mouse model for Gitelman's syndrome. *J Am Soc Nephrol* 2004; 15:2276-2288

[10] Xi Q, Hoenderop JGJ, Bindels RJM. Regulation of magnesium reabsorption in DCT. *Pflugers Arch* 2009; 458:89-98

[11] Brooks H, Sorensen A, Terris J, et al. Profiling of renal tubule Na+ transporter abundances in NHE3 and NCC null mice using targeted proteomics. *J Physiol* 2001; 530: 359-366

[12] Morris RG, Hoorn EJ, Knepper MA. Hypokalemia in a mouse model of Gitelman's syndrome. *Am J Physiol Renal Physiol* 2006; 290:1416-1420

[13] Loffing J, Loffing-Cueni D, Hegyi I, et al. Thiazide treatment of rats provokes apoptosis in distal tubule cells. *Kidney Int* 1996; 50:1180-1190

[14] Breton S, Alper SL, Gluck SL, Sly WS, Barker JE, Brown D. Depletion of intercalated cells from collecting ducts of carbonic anhydrase II-deficient (CAR2 null) mice. *Am J Physiol Renal Physiol* 1995; 269:F761-774

[15] Brion LP, Suarez C, Saenger P. Postnatal disappearance of type A intercalated cells in carbonic anhydrase II-deficient mice. *Pediatr Nephrol* 2001; 16:477-481

[16] Weibel ER. Stereological Methods: Practical Methods for Morphometry. *Academic Press* 1979

[17] Loffing J, Loffing-Cueni D, Valderrabano V, et al. Distribution of transcellular calcium and sodium transport pathways along mouse distal nephron. *Am J Physiol Renal Physiol* 2001; 281:F1021-F1027

[18] Schmitt R, Ellison DH, Farman N, et al. Developmental expression of sodium entry pathways in rat nephron. *Am J Physiol* 1999; 276(3):F367-381.

[19] Reilly RF, Ellison DH. Mammalian Distal Tubule: Physiology, Pathophysiology, and Molecular Anatomy. *Physiol Rev* 2000; Vol. 80, No. 1

[20] Kleyman TR, Kashlan OB, Hughey RP. Epithelial Na+ Channel Regulation by Extracellular and Intracellular Factors. *Annu Rev Physiol* 2018; 80:263-281.

[21] Sorensen MV, Grossmann S, Roesinger M, et al. Rapid dephosphorylation of the renal sodium chloride cotransporter in response to oral potassium intake in mice. *Kidney Int* 2013; 83:811-824

[22] U.S. Department of Agriculture, Agricultural Research Service. What We Eat in America, NHANES 2015-2016; downloaded on 2019-03-23 from: https://www.ars.usda.gov/ARSUserFiles/80400530/pdf/1516/Table_1_NIN_GEN_15.pdf

[23] Nijenhuis T, Vallon V, van der Kemp A, Loffing J, Hoenderop JGJ, Bindels RJM. Enhanced passive Ca2+ reabsorption and reduced Mg2+ channel abundance explains thiazide-induced hypocalciuria and hypomagnesemia. *J Clin Invest* 2005; 115(6):1651-1658

[24] Costanzo LS, Windhager EE. Calcium and sodium transport by the distal convoluted tubule of the rat. *Am J Physiol* 1978; 235(5):F492-506.

[25] Gesek FA, Friedman PA. Mechanism of calcium transport stimulated by chlorothiazide in mouse distal convoluted tubule cells. *J Clin Invest* 1992; 90(2):429-38.

[26] Hollifield JW. Thiazide treatment of systemic hypertension: effects on serum magnesium and ventricular ectopic activity. *Am J Cardiol* 1989; 63(14):22G-25G

[27] Kuller L, Farrier N, Caggiula A, Borhani N, Dunkle S. Relationship of diuretic therapy and serum magnesium levels among participants in the Multiple Risk Factor Intervention Trial. *Am J Epidemiol* 1985; 122(6):1045-59

[28] Lin SH, Cheng NL, Hsu YJ, Halperin ML. Intrafamilial phenotype varibility in patients with Gitelman Syndrome having the same mutations in their Thiazide-sensitive sodium/chloride cotransporter. *Am J Kidney Dis* 2004; 43:304-12

[29] Conti G, Vitale A, Tedeschi S, et al. Hypokalaemia and failure to thrive: report of a misleading onset. *J Paediatr Child Health* 2010; 46:276-277

[30] Tammaro F, Bettinelli A, Cattarelli D, et al. Early appearance of hypokalemia in Gitelman syndrome. *Pediatr Nephrol* 2010; 25:2179-2182

[31] Wagner CA, Loffing-Cueni D, Yan Q, et al. Mouse model of type II Bartter's syndrome. II. Altered expression of renal sodium- and water-transporting proteins. *Am J Physiol Renal Physiol* 2008; 294:F1373-F1380

[32] Rubera I, Loffing J, Palmer LG, et al. Collecting duct-specific gene inactivation of alphaENaC in the mouse kidney does not impair sodium and potassium balance. *J Clin Invest* 2003 112:554-565

[33] Van der Hagen EA, Lavrijsen M, van Zeeland F, et al. Coordinated regulation of TRPV5-mediated Ca^{2+} transport in primary distal convolution cultures. *Pflugers Arch* 2014; 466(11):2077-2087

Tables

Table 1 | Plasma electrolytes in NCC wt and NCC ko mice at age 10 days and 6 weeks

Plasma Na^+ (mmol/l), K^+ (mmol/l), Ca^{2+} (mmol/l) and Mg^{2+} (mmol/l) in NCC wt versus NCC ko mice at the age of 10 days (n = 11) and 6 weeks (n = 5 for Na^+, K^+ and Ca^{2+} and n = 3 for Mg^{2+}). Values are means ± SD. Plasma Mg^{2+} (mmol/l) in NCC wt versus NCC ko mice at the age of 6 weeks reveals significant hypomagnesemia in NCC ko mice (* P = 0.00007).

	10d		***6w***	
	NCC wt	**NCC ko**	**NCC wt**	**NCC ko**
Plasma Na^+ (mmol/l)	141.0 ± 15.78	140.1 ± 21.36	147.3 ± 2.06	147.6 ± 1.81
Plasma K^+ (mmol/l)	7.85 ± 1.65	8.54 ± 1.32	3.68 ± 0.30	3.78 ± 0.25
Plasma Ca^{2+} (mmol/l)	2.53 ± 0.25	2.59 ± 0.51	1.13 ± 0.16	1.15 ± 0.11
Plasma Mg^{2+} (mmol/l)	0.96 ± 0.15	0.97 ± 0.09	1.07 ± 0.03	0.75 ± 0.06 *

Figure legends

Figure 1 | Renin gene expression and plasma aldosterone

A Gene expression of renin in kidneys of NCC wt versus NCC ko mice at the age of 1 day, 4 days, 10 days and 6 weeks analyzed by RT-qPCR. Significant difference in NCC wt versus NCC ko at age 1d (* P = 0.0002). Values are means ± SEM. n = 3. **B** Plasma aldosterone (pg/ml) in NCC wt versus NCC ko mice at the age of 10 days after birth analyzed by ELISA assay with significant difference (* P = 0.0097). Values are means ± SEM. n = 4.

Figure 2 | Fractional cortical volume of DCT and NCC mRNA and protein abundance

A NaCl cotransporter (NCC) protein abundance in kidneys of NCC wildtype mice at different ages (1 day, 4 days, 10 days and 6 weeks after birth). **B** Fractional cortical volume (%) of whole DCT in NCC wt mice. DCT were identified due to NCC immunostaining. Significant difference in NCC wt (d1) ***** P = 0.002 versus NCC wt (6w). Values are means ± SEM. n = 3. **C** Gene expression of NaCl cotransporter NCC in kidneys of NCC wt mice at different ages (1 day, 4 days, 10 days and 6 weeks after birth) analyzed by RT-qPCR. Significant difference in NCC wt (d1) ***** P = 0.0002 versus NCC wt (6w) and NCC wt (d4) ***** P = 0.002 versus NCC wt (6w). Values are means ± SEM. n = 3. **D** Protein expression of NaCl cotransporter NCC in total kidney homogenates of NCC wt mice at different ages (1 day, 4 days, 10 days and 6 weeks after birth) probed on Western blot. Significant difference in NCC wt (d1) ***** P = 0.0006 versus NCC wt (6w) and NCC wt (d4) ***** P = 0.0145 versus NCC wt (6w). Values are means ± SEM. n = 3.

Figure 3 | DCT1-DCT2 ratio at different ages

Percentage of fractional cortical volume of DCT1 (based on parvalbumin immunostaining) and DCT2 with respect to entire DCT fractional cortical volume (based on NCC immunostaining) in NCC wt mice at different ages (1 day, 4 days, 10 days and 6 weeks). Values are means ± SEM. n = 3 (1d, 4d), n = 4 (10d), n = 6 (6w).

Figure 4 | Fractional cortical volume of DCT1 and parvalbumin mRNA abundance

A Parvalbumin (PV) protein expression in kidneys of NCC wt mice at different ages (1 day, 4 days, 10 days and 6 weeks after birth). **B** PV protein expression in kidneys of NCC ko mice at different ages. **C** Fractional cortical volume of DCT1 (%) in NCC wt versus NCC ko mice at different stages of development (1 day, 4 days, 10 days and 6 weeks after birth). DCT1 were

identified due to PV immunostaining. Significant differences in NCC ko compared to age-corresponding NCC wt at age of 1 day (* P = 0.005), 10 days (* P = 0.005) and 6 weeks (* P = 0.00004). Values are means ± SEM. n = 3. **D** Gene expression of parvalbumin in NCC wt and NCC ko mice. Significant difference in NCC ko (6w) ***** P = 0.00004 versus NCC wt (6w). Values are means ± SEM. n = 3.

Figure 5 | DCT1 cell proliferation in NCC wt and NCC ko mice at age 4 days and 10 days evaluated by PCNA immunodetection

A Double-immunostaining for parvalbumin (PV) to identify DCT1 segments (D1) and proliferating cell nuclear antigen (PCNA) to identify proliferating cells in paraffin kidney sections of 4-days-old NCC wt and NCC ko mice. **B** Double-immunostaining for PV and PCNA in 10-days-old NCC wt and NCC ko mice. **C** PCNA-positive DCT1 cell nuclei within kidney cross sections of 4-days and 10-days-old NCC wt versus NCC ko mice expressed as percentage of all DAPI-stained cell nuclei in the DCT1 segments. Significant differences in NCC ko (4d) versus NCC wt (d4) ***** P = 0.0002 and NCC ko (10d) versus NCC wt (10d) ***** P = 0.0001. Values are means ± SEM. n = 3 (4d, 10d).

Figure 6 | TRPM6, TRPV5, α-ENaC, β-ENaC and ɣ-ENaC protein abundance

A Protein expression of TRPM6, TRPV5, α-ENaC, β-ENaC and ɣ-ENaC in total kidney homogenates of NCC wt and NCC ko at different ages (1 day, 4 days, 10 days and 6 weeks after birth). **B** Difference in protein expression (%) of NCC ko at different ages (1 day, 4 days, 10 days, 6 weeks) compared to average of NCC wt of age-corresponding group. Day 1: Significant difference in NCC ko (d1) versus NCC wt (d1) for TRPM6 (***** P = 0.022). Day 4: Significant difference in NCC ko (4d) versus NCC wt (d4) for TRPM6 (***** P = 0.0002), α-ENaC cleaved (***** P = 0.012), β-ENaC (***** P = 0.013) and ɣ-ENaC (***** P = 0.0075). Day 10: Significant difference in NCC ko (10d) versus NCC wt (10d) for TRPM6 (* P = 0.0014), α-ENaC cleaved (* P = 0.023), β-ENaC (* P = 0.016), ɣ-ENaC (* P = 0.012) and ɣ-ENaC cleaved (* P = 0.008). 6 weeks: Significant difference for NCC ko (6w) versus NCC wt (6w) for TRPM6 (* P = 0.002) and ɣ-ENaC cleaved (* P = 0.000035). Statistical analysis was performed combining the densitometry results obtained from two independent western blots (figure 6A and supplementary data, figures S4+S5). Values are means ± SEM. n = 3 (1d, 4d), n = 9 (10d, 6w).